ITALIAN CHEESE RIPENING. IV. VARIOUS FREE AMINO AND FATTY ACIDS IN COMMERCIAL PROVOLONE CHEESE[1,2]

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A need for more complete information on the complex ripening process of cheese is now well recognized, and research is proceeding in this direction. To this end, determinations of specific end-products of cheese ripening have been given attention in recent years by several investigators *(5, 7, 8, 11)*. However, most of these studies are limited in that they have involved measurements of a single class of chemical compounds, e.g., either protein degradation or fat degradation products, whereas the characteristics of ripened cheese result from complex chemical interrelationships. Therefore, a complete knowledge of ripening changes cannot be obtained until studies of a broad nature are conducted in which individual compounds of various classes are considered simultaneously.

General information concerning the protein and fat degradation in the ripening of Italian cheese has been reported earlier *(3, 4)*. This paper presents the results obtained in a study dealing specifically with the identification and measurement of various fatty acids and amino acids in Provolone cheese.

PROCEDURE

Two phases were involved in this investigation. The first phase was concerned with the analysis of ten commercial Provolone cheeses at 9 months of age for various free amino and fatty acids. The second phase involved analysis for free butyric and glutamic acids of 20 commercial Provolone cheeses of various ages and of different intensities of characteristic flavor.

The cheeses of the same age were manufactured by various commercial companies, each using its own specific method. The manufacture of each lot of cheese was observed and complete manufacturing data were obtained by one of the authors.[3] Although these cheeses were manufactured at different factories, the processes were similar. The principal variables were the type and source of enzyme preparation and the starter culture. In this paper the term enzyme preparation means the rennin and lipase-containing preparation(s) added to the milk.

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[3] W. J. Harper.

At each plant, samples of starters were collected and sent directly to the Dairy Products Section, Eastern Utilization Research Service, USDA, for bacteriological analyses. The cheeses were transported to The Ohio State University, where they were cured at 50 °F. and 75% relative humidity for 9 months. These were considered to be aged cheeses, and analyses were made of cheese of the same age in order to determine what variations existed in the end-product content of the various samples.

All cheeses were judged organoleptically by a panel of experienced judges. Particular attention was given to the intensity of characteristic flavor development of the cheese.

The cheeses were sampled by removing a 2-in. cross section from the cheese, discarding the outer ¼ in. of rind and grinding the sample with a rotary grater. Moisture and water-soluble nitrogen were determined as outlined previously *(3)*. For amino acid analyses a 100-ml. aliquot of the water extract *(3)* was concentrated to 10 ml. under vacuum (20 mm.) at 5 °C. Antifoam B (Dow-Corning) was used to eliminate foaming. This product did not affect the results of amino acid analyses.

Five grams of cheese were analyzed for acetic, propionic, and butyric acids by the direct chromatographic procedure of Harper *(2)*. This method utilizes a column of buffered, acidic silica gel, and butanol-chloroform mixtures to effect the separation and quantitative elution of these compounds.

The buffered paper chromatographic method of McFarren *(9)* was used to separate the amino acids. Alanine, aspartic acid, glutamic acid, glycine, and threonine were separated by using phenol as the mobile phase and pH 12.0 buffer as the nonmobile phase. Benzyl-butyl alcohol was used as the mobile phase with pH 6.2 buffer for the nonmobile phase in the separation of the leucines. Histidine, α-amino butyric acid, methionine, phenylalanine, and valine utilized M-cresol for the mobile phase and pH 8.4 buffer for the nonmobile solvent. The solvents used as the mobile phases were redistilled prior to each chromatographic analysis to remove impurities, which would affect the results.

Amino acid separations were conducted at a constant temperature of 21° ± 1 °C. Filter paper-lined chambers were used to maintain a constant humidity, and they were equilibrated for 24 hours before beginning the separation. A standard concentration of each individual known acid was chromatographed in all experiments to serve as a control.

Quantitative analysis of the amino acids was achieved by modifying slightly the ninhydrin technique of Moore and Stein *(10)* as applied to paper by Honer and Tuckey *(5)*. All lots of water and alcohol were boiled prior to use in order to eliminate ammonia contamination from interfering in color development. After color development was achieved, dilution was made by the addition of 2.0 ml. of boiled distilled water.

All amino acid analyses were made within 12 hours after the preparation of the water extracts, the extracts being held at –20° C. until used. Tests showed that the free amino acid content of the concentrate did not change during 24 hours of storage at this temperature but did change on longer storage.

Trials were made to establish reliability of the amino acid methods. The R_f values for the pure amino acids were found to be reproducible and to agree with the published values except for histidine and valine. Histidine gave an R_f value of 0.40 ± 0.01, and valine had an R_f value of 0.49 ± 0.01. The R_f value of α-amino butyric acid that was not reported previously *(9)* was found to be 0.34 ± 0.01. In all trials, the R_f values obtained for any amino acid separated from cheese extracts did not vary by more than ± 0.02 R_f. This indicates the improbability of peptide interference—a fact demonstrated for one sample by acid hydrolyzing and rechromatographing the free acids in the chromatogram.

Recovery trials revealed that the cheese extracts did not affect the results of the paper chromatographic analysis, since recovery of added amino acids varied from 91 to 98% for all of the acids studied.

RESULTS AND DISCUSSION

The enzyme preparation, type of starter organism, flavor description, age, moisture, and water-soluble nitrogen contents of the ten samples of 9-month-old commercial Provolone cheeses are given in Table 1.

TABLE 1

Composition of commercial 9-month-old Provolone cheese

Sample No.	Source and type of enzyme preparation	Description of flavor[a]	Moisture	Water-soluble nitrogen as % of total nitrogen	Organism found in starter
			(%)	*(%)*	
1	Domestic calf paste	Cheddar flavor CF-0	40.5	33.15	*L. lactis* mycoderm, yeast
2	Imported calf paste	Mild CF-1	38.0	21.5	*L. bulgaricus* lactose fermenting yeast
3	Domestic calf rennet paste	Mild CF-1	35.7	14.6	*L. bulgaricus* yeast
4	Domestic calf rennet paste	Mild CF-1	38.0	20.8	*L. lactis* mycoderm, yeast
5	Unknown	Mild CF-1	33.2	19.3	*L. bulgaricus*
6	Calf gland enzyme product	CF-2	34.5	13.8	*L. lactis*
7	Kid rennet paste	CF-2	40.2	31.9	*L. lactis* *S. durans*
8	Kid gland enzyme	Piquant CF-3	37.8	23.5	*L. lactis* and lactose fermenting yeast
9	Kid rennet paste	Good CF-3	34.5	17.5	*L. bulgaricus*
10	Kid gland and calf gland enzyme	Sharp, Ideal CF-4	33.9	22.4	*S. thermophilus*

[a] CF = characteristic Provolone flavor; intensity range from 0 to 4.

TABLE 2

Free amino acid content of aged commercial Provolone cheese

Sample No.	Description of flavor[b]	Glutamic acid	Leucines	Valine	Alanine	Aspartic acid	Glycine	Methionine	Histidine	Threonine	Amino butyric
		(mg. per gram cheese, dry weight)[a]									
1	CF-0	14.9	16.0	4.7	8.1	4.4	+	1.3	−	−	−
2	CF-1	3.4	11.5	0.1	1.5	1.2	+	+	−	−	−
3	CF-1	2.9	8.5	3.0	1.7	2.3	0.7	+	−	+	−
4	CF-1	5.7	11.6	4.5	3.8	2.4	0.8	0.9	−	−	−
5	CF-1	3.5	6.2	2.1	1.5	+	0.4	+	−	−	−
6	CF-2	8.0	11.6	6.4	2.4	4.4	1.7	+	−	+	−
7	CF-2	8.8	15.0	2.2	2.1	3.1	+	+	−	−	−
8	CF-3	8.4	10.9	5.3	3.1	4.2	1.4	+	+	+	+
9	CF-3	5.4	10.7	3.9	1.5	5.3	0.8	.89	−	+	−
10	CF-4	8.3	12.1	6.8	3.5	4.6	1.3	.58	−	+	−

[a] + indicates presence of the acid but in quantities $>$ 0.1 mg. per gram solids.

[b] CF = characteristic Provolone flavor; intensity range from 0 to 4.

Results reveal that the flavor of the cheeses varied from Cheddar-like to a sharp piquant characteristic Provolone flavor. These data verify the previous report that the flavor was not related to per cent of water-soluble nitrogen but was definitely related to the type of enzyme preparation used.

The results of the free amino acid determinations are given in Table 2. The separation of leucine and isoleucine was incomplete in some samples; therefore, these acids are reported together as combined "leucines."

Qualitatively, the free amino acid content of the various samples was similar. Histidine, α-amino butyric acid, and threonine were generally not present or were in such low concentrations that quantitative measurement was not possible (0.1 mg. per gram cheese solids). The other amino acids were found in all ten samples. However, the concentrations of these acids varied greatly among the different samples of cheese of the same age. For example, the glutamic acid content varied from 2.9 to 14.9 mg. per gram of cheese solids, the valine content varied from 0.1 to 6.8 mg., and the alanine concentrations were between 1.5 and 8.1 mg. The concentration of combined leucine and isoleucine showed the least variation, varying from 6.2 to 16.0 mg. per gram solids.

The amino acids were not present as free acids in the cheese in the same ratio as they are found in the original intact casein. Free glutamic and aspartic acids were generally present in a relatively lower concentration and the leucine and glycine in relatively higher concentrations than they appear in casein. The work of Bassett *et al. (1)* indicates that these differences in ratios might be best explained by random protein degradation of the casein accompanied by specific chemical or enzymatic degradation of the amino acids. The formation of tyramine in Cheddar cheese *(6)* and decreases in free glutamic acid and alanine during certain phases of Cheddar cheese ripening *(5)* serve as examples of such changes. Such secondary reactions would be expected to influence accumulation of end-products and, thus, flavor development.

There was no consistent correlation between the total free amino acid content and the intensity of characteristic flavor. The cheese having the least characteristic flavor had the highest total free amino acid content (Sample 1). However, there was generally a direct relationship between the concentrations of free glutamic acid, alanine, valine, aspartic acid and flavor intensity. For example, the four cheeses with a CF-1 flavor rating contained an average of 3.3 mg. of glutamic acid, 2.4 mg. of valine, and 1.5 mg. of aspartic acid, whereas the five cheeses with a CF-2 or more had an average of 7.6 mg. of glutamic acid, 4.8 mg. of valine, and 4.3 mg. of aspartic acid per gram solids.

The variations in free amino acid content could not be related to the type of rennet paste used in the manufacture of the Provolone cheese. In contrast, a general relationship was observed between the free amino acid content of the cheese and the major lactic acid starter organisms present in the starter culture. Although the samples are too few to give conclusive evidence, the data in Table 3 reveal the average free amino acid content of cheese made with *Lactobacillus lactis* to be much higher than when *L. bulgaricus* was the major starter organism. Tittsler *(12)* reported that *L. bulgaricus* was not found in the cheese after brine

TABLE 3

Relationship of starter organisms to the concentration of free amino acids in commercial Provolone nine months of age

Amino acid	*Lactobacillus lactis*			*Lactobacillus bulgaricus*		
	No. of samples	Concentration of free free amino acids in mg/g solids		No. of samples	Concentration of free free amino acids in mg/g solids	
		Range	Average		Range	Average
Glutamic acid	5	5.7-14.9	9.2	4	2.9-5.4	3.8
Aspartic acid	5	2.4-4.4	3.7	4	0.1-5.3	2.2
Leucines	5	10.9-16	11.0	4	6.2-10.7	9.22
Valine	5	2.2-6.4	4.6	4	0.1-3.9	2.22
Alanine	5	2.4-8.1	3.9	4	1.5-2.4	1.6

saltings, whereas *L. lactis* was found to persist during the ripening period. This possible relationship between the type of starter organism and the accumulation of free amino acids is receiving further study.

Free fatty acids. The free acetic, propionic, and butyric acid content of the ten samples of Provolone are given in Table 4. The butyric acid content varied from 0.18 to 4.2 mg. per gram of cheese solids, whereas the free acetic acid ranged from 0.16 to 0.47. The type of starter could not be related to the free fatty acid content of the different cheese samples. Of the three acids considered, only the free butyric acid was definitely related to the type enzyme product used in making the cheese. The domestic rennet pastes, which generally have been purified, are associated with the less-flavored cheeses and with low free butyric acid values.

The acetic and propionic acids were related to the characteristic flavor of the Provolone cheeses in that two samples criticized as being "acidy" contained the highest concentration of acetic acid. The concentration of free butyric acid

TABLE 4

The lower fatty acid content of aged commercial Provolone cheese

Sample No.	Enzyme product	Flavor	Acetic	Propionic	Butyric
			(mg. per gram cheese solids)		
1	Purified calf rennet paste	Cheddar-like, CF-0[a]	0.16	0.09	0.18
2	Purified calf rennet paste	Mild, acidy, CF-1	0.19	0.22	1.05
3	Imported calf rennet paste	Mild, CF-1	0.18	0.11	1.731
4	Purified calf rennet paste	Mild, CF-1	0.19	0.66	0.50
5	Unknown	Mild, CF-1	0.16	0.15	0.581
6	Glandular calf enzyme product	Good, CF-2	0.16	0.13	1.28
7	Kid rennet paste	Good, CF-2	0.19	0.16	2.496
8	Kid glandular enzyme	Piquant, CF-3	0.13	0.21	3.1
9	Kid rennet paste	Sharp, CF-3	0.22	0.20	2.33
10	Glandular calf and glandular kid enzyme products	Sharp, sl. acidy	0.47	0.25	4.34

[a] CF is intensity of characteristic Provolone cheese flavor; none is CF-0, maximum is CF-4.

TABLE 5

Relationship of characteristic flavor to the free butyric and free glutamic acid content of Provolone cheese

CF[a]-0		CF-0-1		CF-1		CF-2		CF-3		CF-4	
Butyric acid	Glutamic acid	Butyric acid	Glutamic acid	Butyric acid	Glutamic acid	Butyric acid	Glutamic acid	Butyric acid	Glutamic acid	Butyric acid	Glutamic acid
(mg. per gram cheese solids)											
0.19	14.00	1.6	1.4	2.2	2.1	1.5	4.4	2.6	5.8	4.4	8.3
0.51	0.68	1.8	1.1	1.0	3.4	2.6	3.7	2.3	4.0	5.1	10.8
0.55	1.30	1.3	1.9	1.6	2.8	2.7	8.8	3.1	8.4		
0.55	4.1			1.7	2.0	1.2	8.0	2.8	5.4		
0.58	1.60			0.8	5.7	2.1	4.3				
0.88	0.78			0.58	3.5	2.5	8.8				
1.10	0.51										
1.30	0.51										
1.60	0.50										

[a] CF = characteristic flavor. Recorded from 0 to 4.

was found to be directly related to the intensities of the desired flavor of the cheese, with cheese having higher butyric acid values generally having the high characteristic flavor scores. Samples 2 and 3 were exceptions to this relationship.

The interrelationship between butyric and glutamic acid and flavor of Provolone cheese. A possible interrelationship between the concentrations of free butyric and free glutamic acids and the intensity of characteristic flavor was suspected on the basis of the values presented in Tables 2 and 4. In general, the concentrations of both acids were much greater in cheeses that had high flavor scores than in those that had a low flavor score.

In order to study further a possible interrelationship between butyric and glutamic acids, 20 additional samples of Provolone cheese were analyzed for butyric acid and glutamic acid. These cheeses were of various ages from 30 days to 18 months. Each cheese was judged organoleptically. In Table 5 the butyric and glutamic acid values for these 20 samples and the 10 samples previously studied are arranged according to the flavor intensity of the cheese.

The data indicate a definite relationship between the concentration of the free glutamic and butyric acid and the intensity of characteristic flavor of the cheese. The concentration of each of the acids apparently had to reach a threshold value in concentration before the desired flavor was evident. In cheese with CF-1, this threshold value was approximately 2.0 mg. of glutamic acid and 1.0 mg. of butyric acid. Some samples with a CF-0 contained concentration of butyric acid greater than 1.0 mg. per gram, but the glutamic concentration was below the threshold value of 2.0 mg. Two cheeses with more than 2.0 mg. of glutamic acid contained less than 1.0 mg. of butyric acid and possessed no desired flavor. The cheeses with a CF-0 and CF-1 contained a concentration of butyric acid greater than the threshold value, but the glutamic acid concentration was slightly below 2.0 mg.

Cheeses with a CF-2 contained at least 3.5 mg. of glutamic acid and 1.5 mg. of butyric acid. This is approximately a 2:1 ratio. At least 4.0 mg. of glutamic acid and 2.3 mg. of butyric acid were present in cheese with a CF-3. The two cheeses with a CF-4 contained the highest concentration of butyric acid and relatively high concentrations of free glutamic acid. Here again the ratio of free glutamic to free butyric acid was approximately 2:1.

SUMMARY

The free amino acid and free fatty acid content of commercial Provolone cheeses of the same age exhibited wide variations. The free fatty acid content varied in relation to the type of enzyme product used in its manufacture, whereas the free amino acid content was apparently related to the type of starter organism.

Purified glandular lipases were found to produce cheese of at least comparable quality to that made with crude rennet pastes. The importance of the lipase in these enzyme products has been shown to be related directly to the production of butyric acid.

An interrelationship was noted between the concentration of free butyric acid and free glutamic acid (per gram of cheese solids) and desired flavor intensity. About 1 mg. of free butyric acid and 2 mg. of free glutamic acid were found in cheeses in which characteristic flavor was noted. The concentration of each increased with flavor intensity, maintaining a ratio of approximately 1:2 of butyric to glutamic acid.

The results of this study emphasize the value of measuring individual degradation products of both fats and proteins simultaneously. More complete analyses for various types of compounds appear to be a valuable approach for future studies.

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